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Sarah Wilcox
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Denise L. Faustman	Confirmation No.:	3056
Serial No.:	10/698,734	Art Unit:	1644
Filed:	October 31, 2003	Examiner:	Michail A. Belyavskyi
Customer No.:	21559		
Title:	METHODS OF ORGAN REGENERATION USING HOX11-EXPRESSING PLURIPOTENT CELLS (As Amended)		

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF DENISE FAUSTMAN, M.D., Ph.D.

I declare:

1. I am the named inventor of the subject matter described and claimed in United States Patent Application Serial No. 10/698,734 (the “734 application”), which was filed on October 31, 2003.

2. I am an Associate Professor of Medicine at Harvard Medical School and Director of the Immunobiological Laboratories at the Massachusetts General Hospital. I am also a member of the American Association for the Advancement of Science and co-editor in chief of the Journal of Women's Health. In addition, I am a senior author of over 100 peer-reviewed publications in internationally recognized scientific journals.

3. I have read and understood the Office Action mailed on March 12, 2007. This Declaration is presented to overcome the rejection of claims 1-12, 30, 45, 46, 53-57, 59, and 60 under 35 U.S.C. § 112, first paragraph, for lack of enablement. I have considered the Office's remarks regarding the teachings of the specification with respect to the scope of the present claims.

4. A discovery that formed a basis for the '734 application was the identification of a previously unknown reservoir of *Hox11*-expressing pluripotent cells in the capsule of the normal spleen. The *Hox11*-expressing cells are not found in other hematopoietic tissues, such as bone marrow and thymus, and are not found in other examined peripheral organs. I discovered that the *Hox11*-expressing cells are capable of self-renewal and have multilineage regenerative potential. As is discussed in more detail below, I have shown that *Hox11*-expressing cells, when administered to a mammal, engraft within injured, damaged, or deficient organs and tissues and differentiate into functional cells within the organs and tissues or promote the regeneration of endogenous functional cells within the organs and tissues. In particular, I observed that *Hox11*-expressing cells differentiate into, or increase or maintain, endogenous insulin-secreting beta islet cells, salivary epithelial cells, and inner ear cochlear cells. In addition, I observed that the administration of *Hox11*-expressing cells to mammals having injured, damaged, or deficient organs and tissues as a result of, e.g., autoimmune disease, restores self-tolerance and, when administered with TNF- α , a TNF- α inducing substance, or a TNF- α agonist, eliminates autoreactive lymphocytes, thereby promoting the regrowth of damaged tissues and organs and reversing autoimmune disease in the mammal.

5. The data presented in the specification and in this declaration were obtained using an NOD mouse model, which is an accepted animal model of type 1 (autoimmune) diabetes mellitus,

Sjögren's syndrome, and lupus in humans. The NOD mouse mimics the autoimmune destruction of functional cells in the organs and tissues of patients with autoimmune disease, e.g., beta islet cells in the pancreas of human patients with type I diabetes and epithelial cells in the salivary gland of patients with Sjögren's syndrome. Thus, the NOD mouse is a valuable model for demonstrating the beneficial effect of introduced adult *Hox11*-expressing cells in regenerating injured, damaged, or deficient organs or tissues.

6. The data reported in the specification provide evidence that the administration of *Hox11*-expressing cells to a mammal increases or maintains functional cells in a mammal having an organ or tissue that is injured, damaged, or deficient in the functional cells. In particular, the present specification presents data showing that *Hox11*-expressing cells restored normoglycemia in 6 out of 9 diabetic NOD mice that were administered the cells in a first study, and in 11 out of 12 diabetic NOD mice administered the cells in a second study (see Examples 1 and 2, pages 29-33 of the specification). The specification further teaches that the pancreata of the NOD mice that received the *Hox11*-expressing cells exhibited the reappearance of pancreatic islets without invasive insulitis and with minimal or no peri-insulitis (Specification, p. 33, lines 6-8). In Example 3, the specification describes evidence of chimerism in the NOD mice administered *Hox11*-expressing cells, which confirms that these cells remain in the host mammal after administration and regenerate the pancreas by increasing or maintaining beta islet cells (Specification, p. 34, line 23, through page 35, line 2). Finally, Example 4 of the specification confirms that the administration of donor *Hox11*-expressing cells to five NOD mice which lacked detectable islets before treatment resulted in the regeneration of beta islet cells in the pancreas (Specification, p. 36, line 23, through page 37, line 2). Moreover, 29% to 79% of the islet cells observed in these NOD mice were of donor origin; none of the islets cells were solely host-derived. This further confirms that the *Hox11*-expressing cells contributed directly to islet regeneration. Thus, the present specification clearly teaches, and provides experimental evidence that confirms, that the administration of *Hox11*-expressing cells to a mammal having an injured, damaged, or deficient organ or tissue, in this case a damaged pancreas, increases the number of functional cells

in the mammal, here beta islet cells of the pancreas, reverses invasive insulinitis, and restores normoglycemia.

7. Researchers working under my direction conducted additional experiments that support and further validate the data reported in the specification. These experiments demonstrate that the methods of the invention can be used to increase or maintain functional acinar epithelial cells that are injured, damaged, or deficient in NOD mice exhibiting symptoms characteristic of Sjögren's syndrome by administering *Hox11*-expressing cells to these mice.

Similar to disease in humans, NOD mice develop histopathological and functional changes in their salivary glands due to autoimmune attack on the acinar epithelial cells of the salivary gland; the salivary glands display infiltrates of lymphocytes and a gradual loss of salivary function, which is observed as a reduction in saliva output. We observed that the administration of *Hox11*-expressing cells to NOD mice increased or maintained the number of acinar epithelial cells in the injured, damaged, or deficient salivary glands of these mice, which restored near normal levels of saliva secretion.

In particular, we monitored the salivary flow rates (SFR) of thirteen female NOD mice exhibiting characteristics of advanced Sjögren's syndrome (i.e., greater than 50% loss of salivary flow). The mice were randomized into two groups: untreated (n=5) versus treated (n=8). All NOD mice were normoglycemic at the start of therapy (14-week-old). Treated female NOD mice received one injection of CFA for TNF induction and male donor live MHC class I and peptide matched *Hox11*-expressing splenocytes bi-weekly for 40 days. The use of male *Hox11*-expressing splenocytes allowed for post-transplantation tracking of these Y-chromosome cells (using fluorescence *in situ* hybridization (FISH)) in female NOD salivary and pancreatic tissue.

Female NOD mice exhibited a decrease in SFR at 12-14 weeks of age. SFR were compared between untreated NOD, treated NOD, and age-matched C57BL/6 (normal) mice at 10 weeks (prior to Sjögren's syndrome onset), 14 weeks (prior to CFA/splenocytes treatment), 21 weeks (completion of treatment), and finally at 35 weeks of age (animals were sacrificed and their tissues harvested). All eight treated NOD mice exhibited a decrease in SFR during the 40 days of active therapy. During the next 120 days, however, there was a gradual restoration of SFR

(Fig.1A). By 160 days, SFR of treated NOD mice was comparable to that of age-matched C57BL/6 mice ($p=0.6434$). Treated NOD were also protected from diabetes ($p=0.0002$; Fig.1B). The SFR of untreated NOD mice continued to deteriorate over time, and all of them died of severe hyperglycemia within 140 days from the start of therapy. SFR directly reflects function of the salivary glands and its decrease is the major clinical finding in patients with Sjögren's syndrome. Treatment with CFA and *Hox11*-expressing cells reversed both Sjögren's syndrome (salivary gland) and diabetic autoimmunity (pancreas) with remarkable success in the NOD mice.

We also examined the salivary tissues histologically for chimerism and inflammatory signs. We combined FISH with immunohistochemistry (IHC) on the same section to allow for the simultaneous detection of both the Y chromosome and specific markers for salivary or pancreatic cells. A small number of Y-positive salivary epithelial cells (Fig. 2A-D) and a few pancreatic beta cells were detected in the treated NOD, but not in untreated NOD or C57BL/6 mice. We observed the reappearance of pancreatic islets free of invasive lymphoid infiltrates in treated NOD. However, in salivary glands, the lymphocytic infiltrates (focus score) did not differ significantly between *Hox11* cell-treated and untreated animals ($p=0.23$).

Our data confirm that the administration of *Hox11*-expressing cells, according to the method of present claims 1-12, 30, 45, 46, 53-57, 59, and 60, is not only effective for increasing or maintaining functional cells (i.e., beta islet cells) in the pancreas of diabetic NOD mice, but also increasing or maintaining functional cells (i.e., acinar epithelial cells) in the salivary glands of these mice, thereby reversing established Sjögren-like syndrome in this animal model. Our data show not only 100% protection from progression to diabetes when this therapy was administered to young (14-week-old) NOD mice, but also islet regeneration and rescue. Our data also demonstrate complete restoration of salivary gland function, as measured by SFR. A small number of donor *Hox11*-expressing splenocytes colonized the salivary gland and regenerated this tissue by differentiating into salivary epithelial cells. In addition, we observed endogenous regeneration of acinar epithelial cells following the cellular therapy.

8. Researchers working under my direction also demonstrated that the methods of present claims 1-12, 30, 45, 46, 53-57, 59, and 60 can be used to increase or maintain functional cells of the

inner ear that are injured, damaged, or deficient in a mammal by administering *Hox11*-expressing cells to the mammal. We observed that NOD mice at early stages of post-natal life have complete hearing loss accompanied by structural defects in the development of the inner ear. The introduction of *Hox11*-expressing cells from the spleens of normal adult mice stably restore long-term hearing in these deaf NOD mice by replacing inner ear cells and by stimulating endogenous regeneration of *Hox11*-derived tissues.

Hearing function was measured using auditory brainstem responses (ABRs) in NOD, NOD-*SCID*, and C57BL/6 control mice at three ages: 15, 23 and 33 weeks. NOD-*SCID* mice differ from NOD mice by a single genetic mutation (*scid*) that ablates the immune system and thus prevents autoimmune mediated disease. No measurable hearing was detected in both the NOD and NOD-*SCID* mice when tested at thresholds at or above 100 dB at all frequencies tested (5 to 40 kHz) as compared to C57BL/6 mice (Fig. 3A). The complete hearing loss in the NOD and NOD-*SCID* mice was primary to the animals (i.e., it was not due to secondary factors, such as destruction by autoimmune cells) based on the presence of the defect in both NOD and NOD-*SCID* mice at all time points studied and the fact that NOD-*SCID* mice lack a functional immune system (Fig. 3B). The control mice showed hearing with normal age-related hearing loss over the time-course from 15 to 33 weeks of age (Fig. 3C). Of the animals studied, 100% (13/13) of the NOD mice and 90% (9/10) of the NOD-*SCID* mice had no measurable hearing at the frequencies tested (Fig. 3A and B). Therefore, the presence or absence of an immune system had no measurable impact on the early and complete hearing loss in NOD and NOD-*SCID* mice.

Inner ear structures in the cochlea were compared between NOD and C57BL/6 mice (Figs. 4A-B). Cross-sections of the spiral ganglion, spiral ligament, and organ of Corti were examined and all NOD samples showed deterioration of the spiral ganglion with only Schwann cell nuclei remaining (Fig. 4B(i)-(ii)). Atrophy in the spiral ganglion was found in 100% (18/18) of the NOD cochlea compared to only 22% (2/11) in the control cochlea (Table 1). There was also deterioration in the spiral ligament in the NOD cochlea (Fig. 4B(iii)), which was not present in the control (Fig. 4B(iv)). The spiral ligament appeared abnormal (as defined by at least 50% neuron loss) in 72% (39/54) of the spiral ligaments observed, with no significant difference between the cochlear turns (upper/lower basal, upper/lower second turn, lower third turn). The organ of Corti

(OC) appeared abnormal in 58% (21/36) of the NOD samples with no significant difference between the cochlear turns (Fig. 4B(v)-(vi)). The organ of Corti was considered abnormal if it had more than a 50% loss of inner hair cells or if the organ of Corti had not developed properly, most commonly failing to form the canals in the organ of Corti. Other structural defects were also observed in the NOD cochlea, such as deterioration in the stria vascularis and the spiral limbus of the lower second turn, but both of these defects had a low penetrance. Similar defects were found in the NOD-*SCID* cochlea as in the NOD. Again, as with the hearing data, the similarity between NOD and NOD-*SCID* mice suggest that these functional and morphological abnormalities are primary to the animals and not a secondary outcome of immune-mediated disease.

We injected *Hox11*-expressing cells into 15 week-old NOD mice and evaluated the NOD mice for any gain in hearing function by ABR tests and by histology. Of eight treated NOD mice, two mice (ST-001 and ST-002) showed significant recovery in hearing, while others showed slight increases in hearing, as measured by lower thresholds in the ABR test (Table 2).

The auditory responses and the morphology of the cochlear structures were compared in the two NOD mice, ST-001, ST-002, which received the cellular and autoimmune treatment. Another NOD mouse, ST-012, which received the cellular and autoimmune treatment and exhibited a restoration in glucose levels long-term but no hearing improvement, was also examined. These data were compared to a similarly aged C57BL/6 control (Table 2). The individual audiograms show that the two treated NOD mice, ST-001 and ST-002, showed a return of most low and mid-frequency hearing, while the treated NOD mouse ST-012 showed no measurable hearing recovery throughout the treatment.

In order to determine the effect of the cellular treatment on the NOD mice, we examined the cochlear morphology of the three NOD mice (ST-001, ST-002, and ST-012) that exhibited an improvement in hearing. As seen in Figure 4, the two NOD mice whose diabetic condition and functional auditory responsiveness showed improvement, appeared to have a normal spiral ligament with a full, or nearly full, population of cells in the lower third turn (Fig 4a, c) and lower second turn (Fig 4b, d) of the cochlea. Also, the organ of Corti was restored in both ST-001 and ST-002 with a full population of cells, including hair cells; ST-001 and ST-002 also exhibited restored development of the tunnel of Corti in the lower second and third turns (Fig 4a-d). NOD

mouse ST-012, which exhibited normoglycemia but no improvement in hearing following therapy, showed the continued and expected severe atrophy of cells in the spiral ligament of the lower third turn and lower second turn. This is depicted by a depopulation of cells in the area that corresponds to the spiral ligament in Fig 4e-f. NOD mouse ST-012 did have some apparent improvement in the organ of Corti, i.e., the lower second turn contained a few cells, especially inner and outer hair cells (Fig 4f). The organ of Corti of the lower third turn in the NOD mouse ST-012 exhibited a slightly less severe loss of cells in the lower second turn compared to an untreated NOD mouse, but continued to have the persistent poor development of the tunnel of Corti (Fig 4e) as compared with C57BL/6 control (Fig 4g).

Our histology and hearing function data show that young NOD mice have significant sensory hearing loss due, in part, to structural defects in the inner ear. The defects involve the spiral ganglion as well as loss of the spiral ligament and poor formation of the organ of Corti. The identified abnormal hearing and structures of the inner ear were present in very young mice at 5-12 weeks after birth, which is prior to the typical onset of immune-mediated disease at 20-30 weeks of age. The introduction of normal, *Hox11*-expressing splenocytes to NOD mice permanently removed the underlying autoimmunity and restored hearing function and cochlear morphology. The administered *Hox11*-expressing splenocytes not only regenerated cochlear tissues in the NOD mice by differentiating into cochlear cells and replacing damaged organ tissue with healthy organ tissue, but also promoted endogenous regeneration of cochlear tissues by endogenous cells. Importantly, our data demonstrate that the cellular therapy fully restored functional hearing in 25% of treated NOD mice compared to age matched control mice. Restored partial hearing recovery was observed in another 25% of treated NOD mice.

9. All statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. § 1001 and that such willful false statements may jeopardize the validity of the application and any patents issued thereon.

September 12 2007
Date

Denise Faustman - MD, PhD
Denise Faustman, M.D., Ph.D.

Figure 1

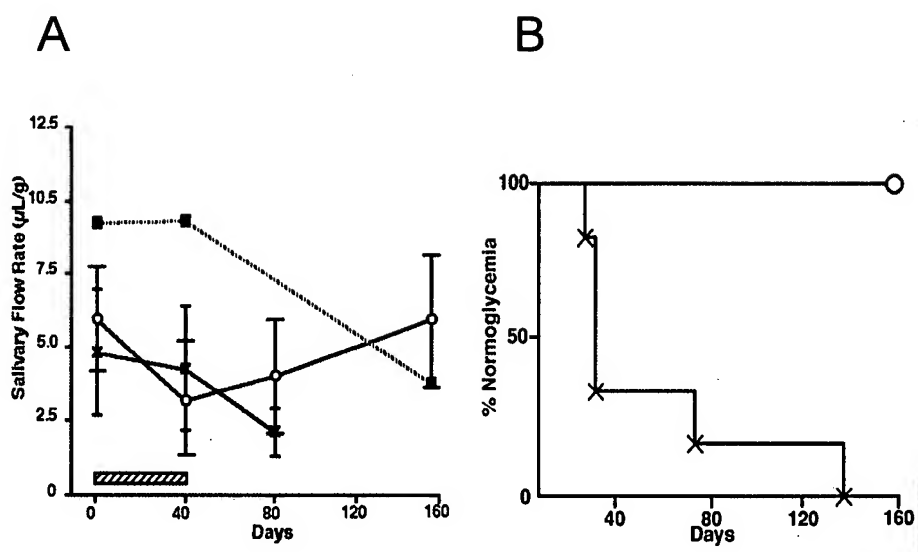


Figure 2

Salivary glands of successfully treated female NOD mice (A- D) are shown. The Y chromosome signal is green and cytokeratin13 (a marker of salivary epithelial cells) is red. Nuclei are stained in blue with DAPI. We observed Y-chromosome positive nuclei in cytokeratin-positive cells of treated female NOD mice. The Y and Z dimensions are shown on the two sides of the X plane image and demonstrate the presence of the Y chromosome (green) in the same plane with the cytokeratin13 (red) and the nucleus (DAPI-blue). These four images (A, B, C, D) were taken from two different treated NOD mice. FISH analysis of salivary tissue sections from control female (E) and male (F) mice are used here as controls for the Y chromosomal probe.

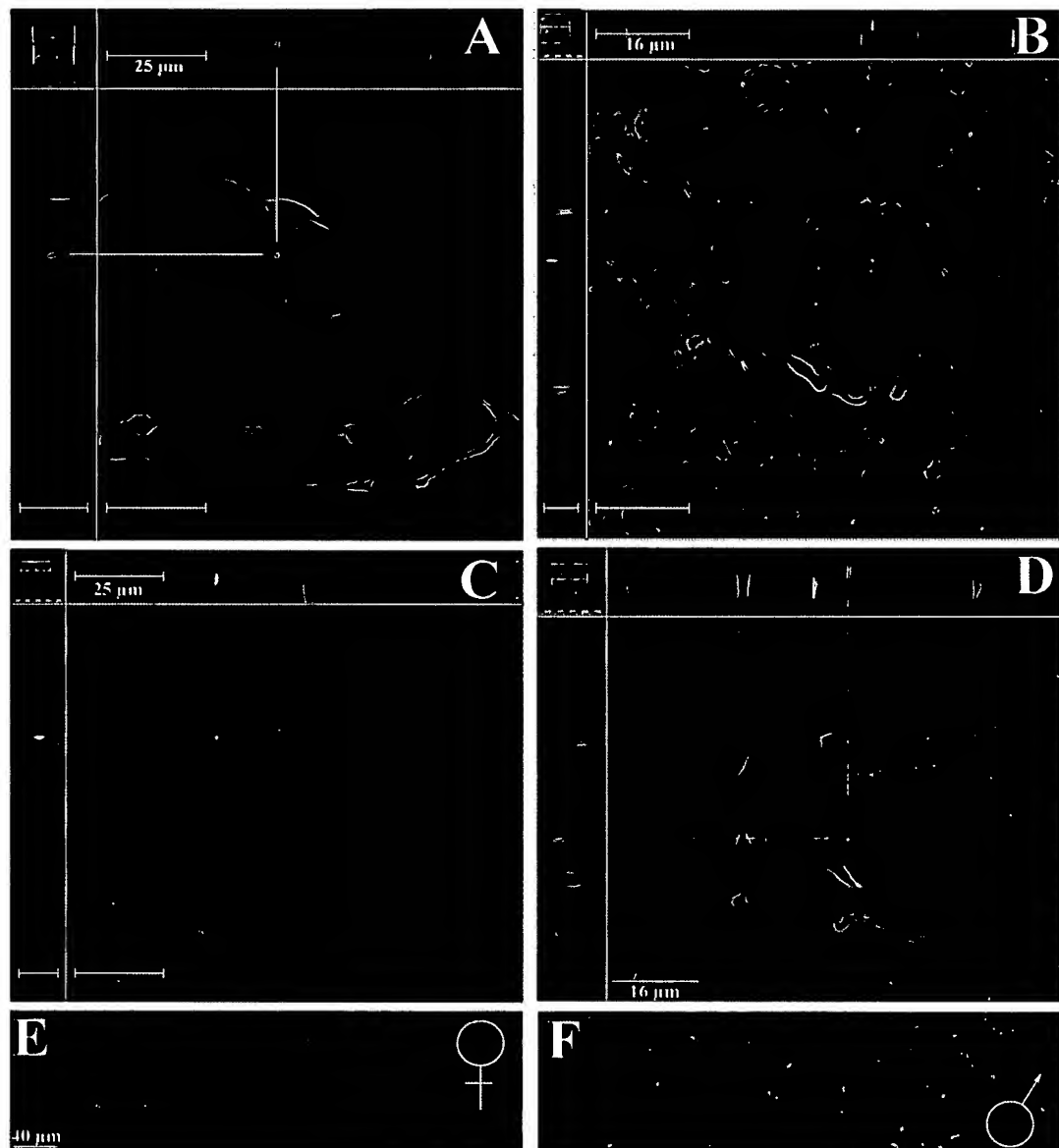


Figure 3

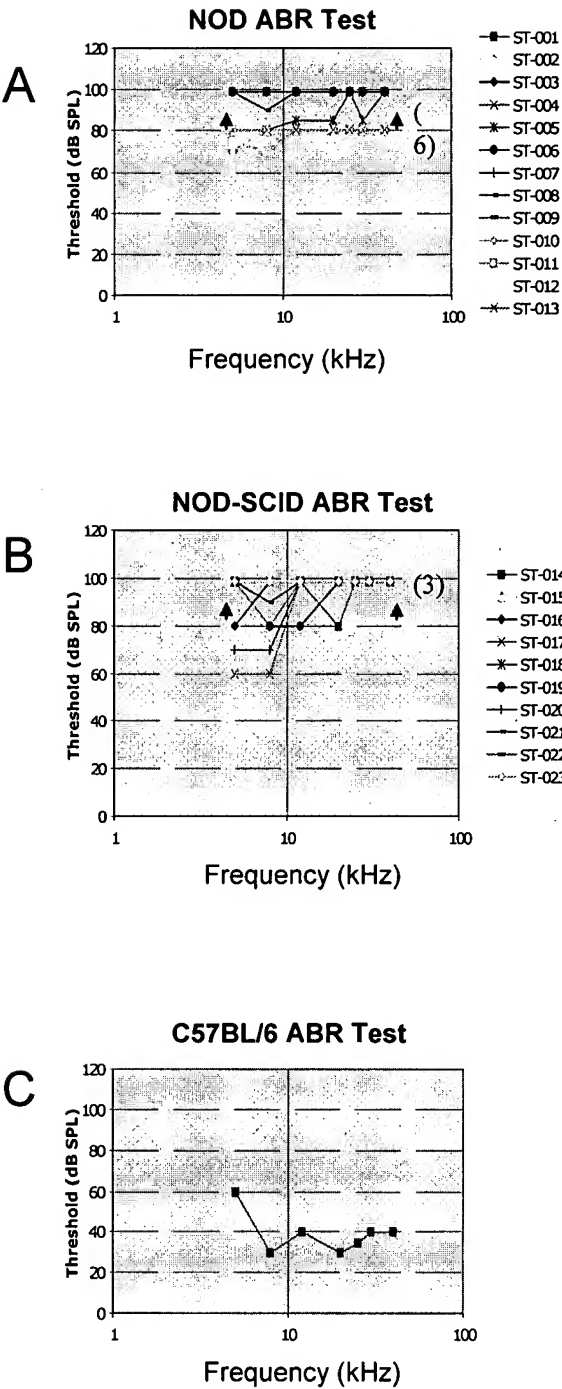


Figure 4

Cochlear morphology of two treated NOD mice with partial restoration of hearing.

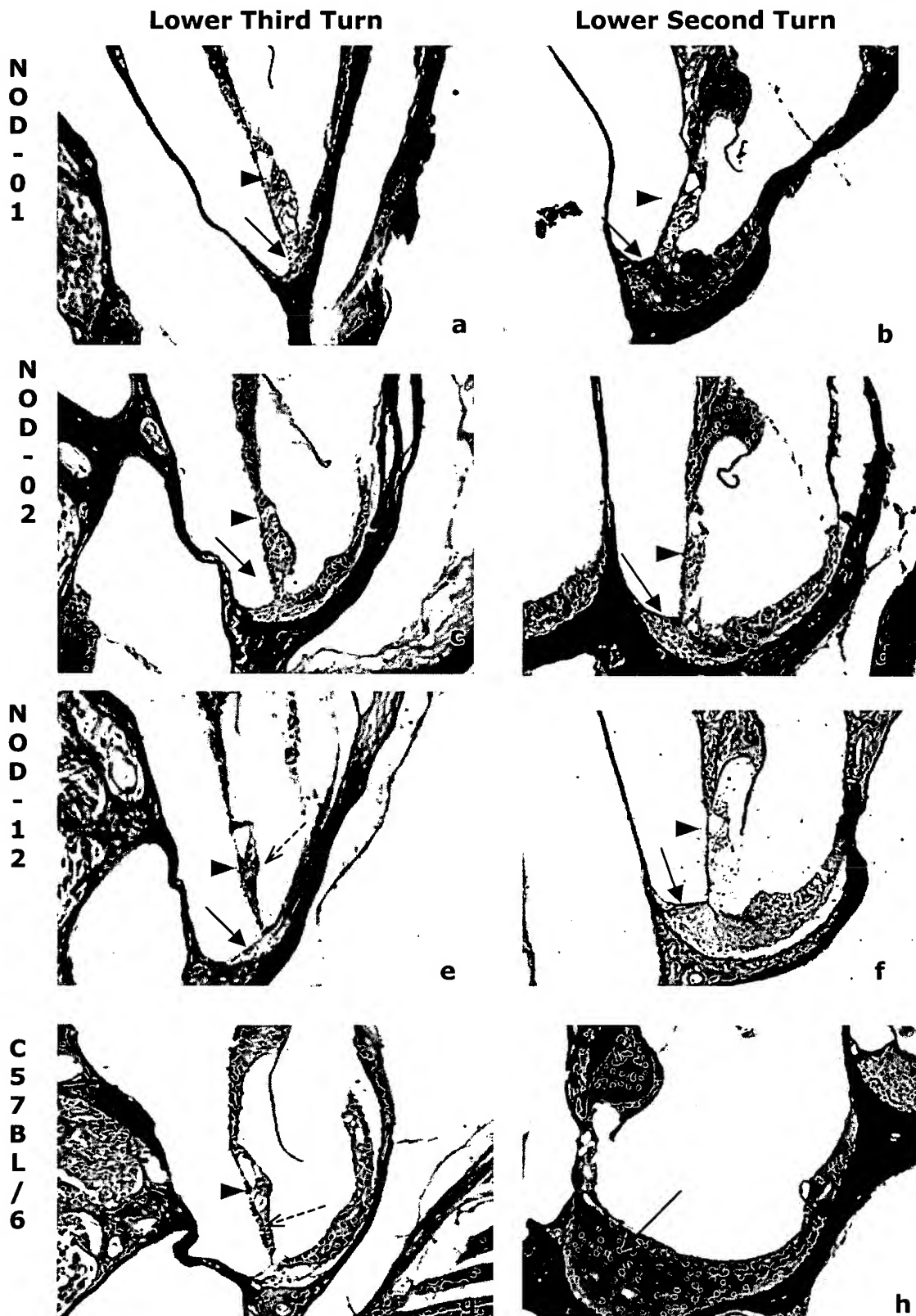


Table 1: Abnormalities in cochlear structures in NOD and NOD-SCID. The organ of Corti (OC) was considered abnormal if it had more than a 50% loss of cells or if the structure had not developed properly, most commonly failing to form the tunnel of Corti. All spiral ganglion were abnormal in the NOD (with a lower rate in the NOD-SCID), where an abnormality in the spiral ganglia was defined by at least 50% neuron loss.

Structure	NOD	NOD-SCID	C57BL/6
Organ of Corti	21/36 = 58%	6/13 = 46%	1/30=3%
Spiral Ligament	39/54 = 72%	7/14 = 50%	5/37=14%
Spiral Ganglia	18/18 = 100%	6/19 = 32%	2/11=22%

Table 2: Functional recovery of hearing in NOD mice with autoimmune treatment. NOD mice were given the autoimmune reversing therapy at 15 weeks of age to prevent progression to diabetes. Of the eight treated NOD mice, all successfully maintained their glucose levels and two had significant recovery of hearing by 33 weeks of age at the low to mid-range frequencies (5 to 25 kHz) as seen be a decrease in hearing thresholds for those frequencies. The C56BL/6 control animals showed no hearing improvement, but did show age-related hearing loss by the age of 33 weeks.

	NOD treated	NOD untreated	C56BL/7
Glucose levels	8/8 = 100% restored	0/5 = 0% restored	No change
Hearing improvement	2/8 = 25%	0/5 = 0%	0/5= 0%